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**Diagnostic and prognostic value of plasma Tumor M2 pyruvate kinase (Tu-M2-PK) in peri-ampullary cancer:
evidence for a novel biological marker of adverse prognosis.**

KS Goonetilleke,
**JM Mason,
HPP Siriwardana,
NKK King,
*MW France,
AK Siriwardena.

Hepato-Pancreato-Biliary Unit and
*Department of Clinical Biochemistry,
Manchester Royal Infirmary
&

**School for Health,
University of Durham, Queen's Campus,
Stockton-on-Tees.

Correspondence to:

Dr AK Siriwardena. MD FRCS

HPB Unit, Department of Surgery
Manchester Royal Infirmary
Oxford Road

Manchester M13 9WL

Tel: 0161-276-4250; Fax: 0161-276-4530

e-mail: ajith.siriwardena@cmmc.nhs.uk

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Abstract

Background: This prospective study examines the diagnostic and prognostic utility of tumor M2 pyruvate kinase (Tu-M2-PK) used in conjunction with CA 19-9 in patients with subsequently histologically confirmed peri-ampullary malignancy.

Methods: Plasma Tu-M2-PK and serum CA 19-9 levels were measured at admission in a cohort of patients with suspected pancreatic cancer. Values for Tu-M2-PK and serum CA 19-9 were compared to a control group comprising jaundiced patients in whom malignancy was excluded by ERCP and non-jaundiced individuals undergoing laparoscopic cholecystectomy.

Results: The mean (sd) plasma Tu-M2-PK level for patients with histologically-proven malignancy was 40.5 (26.4) U/ml and for non-cancer patients was 29.9 (20.9) U/ml (Mann-Whitney U=1163, P = 0.006). Tu-M2-PK had an AOC of 0.623 on ROC analysis and at optimal cut-off of 27 U/ml, sensitivity is 66%, specificity is 58%. However, on multi-variate Cox regression modelling elevated Tu-M2-PK (>27 u/ml) was strongly correlated with the subsequent finding of poorly differentiated cancer and/or metastatic disease and strongly predicted survival on Kaplan-Meier analysis.

Conclusion: An elevated Tu-M2-PK over 27 units/ml measured on admission in suspected peri-ampullary cancer is a predictor of adverse prognosis in peri-ampullary cancer.

(185 words)

Introduction

Pre-operative tissue diagnosis of cancer of the pancreas remains difficult.¹ In those individuals with lesions arising from the ampulla of Vater, pre-operative endoscopic biopsy is feasible.² However, for the majority of patients with pancreatic tumours, current pre-operative diagnosis relies on a combination of interpretative information obtained from imaging techniques such as trans-abdominal ultrasonography, endoscopic ultrasonography (EUS) and computed tomography (CT)³ augmented by information obtained from biopsy, brushings or sampling of pancreatic ductal juice during endoscopic retrograde cholangiopancreatography (ERCP).⁴ To date there is no reliable blood test for the diagnosis of pancreatic cancer.

Sustained searches for a reliable blood test have led to the evaluation of carcinoembryonic antigen (CEA)⁵, carbohydrate antigen (CA) 19-9^{6, 7, 8}, and other tumour-associated antigens such as CA 50⁶, CA 242⁹, foetoacinar protein (FAP)¹⁰ and SPAN-1¹¹ amongst others, either alone or in combination.^{12, 13, 14, 15} Perhaps the best recognised of these is CA 19-9.^{8, 10, 12, 13, 14, 15} Although elevated in cholestasis^{16, 17, 18}, serial sampling after relief of jaundice may be of value¹⁹ but CA 19-9 lacks sufficient discriminant value for use as a sole diagnostic test.^{20, 21}

Recent insights into tumour metabolism have shown that a wide range of human tumours express the enzyme pyruvate kinase, a component of the aerobic glycolysis pathways.²² Of the four different isoforms found in mammalian tissue, the M2 isoenzyme is strongly over-expressed in cancer states and shifts from the tetrameric to the dimeric state which can be detected with a specific monoclonal antibody.²³ A commercially available enzyme-linked immunosorbent assay (ELISA) kit has allowed for the reliable detection and quantification of the enzyme tumour-M2-pyruvate kinase (Tu-M2-PK) in blood.²⁴ Reports suggest that Tu-M2-PK levels may be elevated in patients with breast²⁵, gastrointestinal²⁶, lung²⁷ and renal²⁸ tumours. The reference range for Tu-M2-PK in normal populations is well defined.²⁹

Increasing evidence suggests that Tu-M2-PK may become a valuable plasma marker for the diagnosis of cancer. Oremek and co-workers have established the reference range in 666 consecutive blood donors.²⁹ However it should be noted that Oremek's study was conducted using serum samples which may require a higher cut-off value than EDTA-plasma.²⁴ A cut-off point of 22.5 U/ml in their study corresponded to a sensitivity of 71% with 90% specificity for the detection of cancer. Tu-M2-PK levels are elevated in patients with breast, lung, renal and gastrointestinal cancer.^{25, 26, 27, 28} At least five previous published reports^{26, 29, 32, 33, 34} have evaluated the role of Tu-M2-PK in the diagnosis of pancreatic cancer. Limitations inherent in several previous reports have been the lack of histologic confirmation of malignancy and sufficiently prolonged follow-up to assess survival. The present study follows a cohort of patients referred to a tertiary referral hepato-pancreatico-biliary unit with a provisional diagnosis of pancreatic cancer, and assesses the diagnostic and prognostic utility of Tu-M2-PK.

Methods

Study objective

The purpose of this study was to explore the diagnostic and prognostic value of Tu-M2-PK in patients with peri-ampullary cancer.

Study design

The study was a two-stage prospective, single-centre, clinical evaluation of the diagnostic accuracy of plasma Tu-M2-PK (used either in conjunction with serum CA 19-9 or as a stand-alone test) in patients with subsequently histologically confirmed peri-ampullary cancer. In the first stage, diagnostic performance of Tu-M2-PK (\pm CA 19-9) was compared in confirmed cancer and confirmed non-cancer controls. The influence of jaundice and tumour differentiation was explored as sub-group analyses. In the second stage, the prognostic role of plasma Tu-M2-PK (\pm CA 19-9) was assessed using prospectively recorded survival data (up to 3 years follow-up with a median of 271 [2-1129] days).

Study population

Patients were excluded from either stage if they were unable to give informed consent, were under 16 years of age, if there was subsequent confirmation of a non-pancreatic malignancy or if there was histologically-confirmed concurrent malignancy. Of the non-cancer controls, 23 had jaundice with benign disease being confirmed at endoscopic retrograde cholangiopancreatography (ERCP) and 44 without jaundice had normal enzymatic liver function tests and were undergoing elective laparoscopic cholecystectomy.

Between July 2002 and March 2004, 104 patients were admitted to the hepato-pancreaticobiliary service of the Manchester Royal Infirmary with suspected pancreatic cancer and were eligible for inclusion. Of these patients, 49 had jaundice. Subsequent histopathological findings confirmed pancreatic/peri-ampullary malignancy in 76 patients, of whom 19 had poorly differentiated tumours and 57 had moderately or poorly differentiated tumours. Eighteen patients underwent resection (17%). Seventeen patients had metastatic disease at time of index presentation. The 76 patients with confirmed cancer constitute the disease group for subsequent comparative analyses.

Investigations and principal outcome measures

For the purposes of this study CA 19-9 and Tu-M2-PK levels in blood were assayed at a single time-point prior to resection or pancreatic biopsy in patients with clinically-suspected pancreatic cancer and in patients in the control groups. The principal outcome measures

were estimation of the sensitivity and specificity of Tu-M2-PK (\pm CA 19-9) for the pre-operative diagnosis of peri-ampullary cancer and for prediction of survival.

Sample collection and storage

Following informed consent, a sample of venous blood (3 ml) was drawn into an ethylenediaminetetraacetic acid (EDTA)-containing tube for assay. Samples were centrifuged and supernatant EDTA plasma was separated within 12 hours and stored at -20°C for Tu-M2-PK assay. The relevant serum samples for CA 19-9 were transported to the Department of Biochemistry of the Christie Hospital, Manchester where they were assayed in the regional clinical reference laboratory.

Assay protocols and reference values

Tu-M2-PK levels in EDTA-plasma samples were assayed using a commercially-available ELISA (ScheBo Biotech UK Ltd, Basingstoke, UK)³⁰. The Tu-M2-PK test kit allows the quantification of Tu-M2-PK within the range of 5 to 100 units/ml (U/ml) EDTA plasma. Values out of range are specified as <5U/ml or >100U/ml respectively. The mean intra-assay coefficient of variance (CV) was 3.6% (1.0-8.6%). This compares to the published CV provided by the manufacturer of 3.5% (2.4-7.0%).³⁰ The mean inter-assay CV was 5.4% (3.9-6.5%), compared to the manufacturer's values of 5.3% (3.3-7.5%). The analytical range for the CA 19-9 assay extends from the minimum detectable concentration value of 0.8 to 240 U/ml. Samples with concentrations greater than the upper limit of this analytical range were diluted using a volumetric pipette with Bayer CA 19-9 assay calibrator Level 1 to bring the concentration within the calibration curve and re-assayed.

Ethical approval

The study was approved by the Manchester Research Ethics Committee and registered as a full clinical study with the Research and Development (R&D) office of the Manchester Royal Infirmary.

Data analyses

Data were entered into the Statistical Package for the Social Sciences, version 12.0 (SPSS, Chicago, Illinois, USA) for statistical analysis. Data are presented as median (inter-quartile range and range) and mean (standard deviation).

The Mann-Whitney U test was used to compare assay findings between groups, and the Spearman rho was used for correlations. Youden's index³¹ (sensitivity + specificity - 1) was used to calculate the cut-off value for Tu-M2-PK and CA19-9 that best maximises sensitivity and specificity using the receiver-operating characteristic curve (ROC). The two tests were

compared using the area under the curve (AUC). Exploratory survival analysis utilised stepwise multivariate Cox regression and Kaplan Meier techniques. A probability of less than 0.05 was considered statistically significant.

Results

Plasma Tu-M2-PK (\pm CA 19-9) for diagnosis of peri-ampullary cancer

The mean plasma Tu-M2-PK level for patients with histologically-proven malignancy was 40.5 (26.4) U/ml and for all non-cancer patients was 29.9 (20.9) U/ml (table 1). This difference was statistically significant (Mann-Whitney U=1163, P = 0.006). The mean serum CA19-9 level for patients with histologically-proven malignancy was 2477 (5328) U/ml and for patients in the control groups was 1017 (4011) U/ml (table 1). This difference was also statistically significant (Mann-Whitney U=527, P < 0.001). Summary ROC characteristics are provided in Figure 1. The Tu-M2-PK test with an AOC of 0.623 is unlikely to provide a useful discriminant test for pancreatic cancer. At an optimal cut-off of 27.2 U/ml, sensitivity is 66%, specificity is 58% and the positive likelihood ratio is 1.6. CA 19-9 test with an AOC of 0.74 performs moderately well although unlikely to be an adequate test in isolation. At an optimal cut-off of 39.0 U/ml, sensitivity is 71%, specificity is 74% and the positive likelihood ratio is 2.6.

The Tu-M2-PK test findings appear unaffected by the presence of jaundice in control patients. However, the CA 19-9 test findings differ markedly according to whether jaundice is present (table 2: sub-group analysis). Consequently, CA 19-9 testing may only have some utility in those patients with suspected cancer presenting without jaundice.

Plasma tu-M2-PK and serum CA 19-9 as prognostic markers

The mean plasma Tu-M2-PK level for patients with histologically-proven malignancy was 40.5 (26.4) U/ml and non-confirmed patients was 37.7 (29.3) U/ml. This difference was not statistically significant (Mann-Whitney U=942, P = 0.37). The mean serum CA19-9 level for patients with histologically-proven malignancy was 2477 (5328) U/ml and for patients in the control groups was 1513 (3770) U/ml. This difference was also not statistically significant (Mann-Whitney U=1054, P=0.94).

Sub-group analysis (Table 2) suggested that Tu-M2-PK testing on admission may have prognostic value in identifying individuals with metastatic disease and poorly differentiated tumors. Survival data recorded for patients with confirmed cancer were used to further explore this finding. The correlation structure of putative explanatory variables is shown in Table 3. The correlation between age and gender reflects the older age of female patients (mean age 70 vs. 61). Presence of metastatic disease and identification of a poorly differentiated tumour are strongly correlated. Tu-M2-PK is seen to be correlated with poor differentiation and metastatic disease while CA 19-9 only correlates with metastatic disease and not poor differentiation (p=0.387). Multivariate Cox regressions modelling determinants of survival were built stepwise including age and sex (Table 4). Neither of these variables fitted at conventional levels of statistical significance but are left in subsequent models by

convention. Each of the remaining four explanatory variables was added individually to explore their influence upon survival. Only poor tumour differentiation and Tu-M2-PK test value fitted in the model. Tu-M2-PK was dichotomised at the optimal cut-off (27 ml/U) and fitted as a binary variable in the model. Patients with values above the cut-off were dying 2.6 times faster than those with values below (95%CI: 1.3 to 5.3). A Kaplan Meier survival curve for high and low Tu-M2-PK values is shown in Figure 2. **Figure 3 illustrates a Kaplan meier curve calculated for the subgroup of patients undergoing resection.**

Discussion

Contemporary diagnostic algorithms for pancreatic cancer employ a range of sophisticated imaging and diagnostic modalities. Integration of information from cross-sectional computed tomography together with findings from endoscopic retrograde cholangiopancreatography together with endoscopic ultrasound-guided fine-needle aspiration allows the construction of a logical management plan. In the setting of tertiary care pancreatic surgery, there remains a need for biochemical markers with diagnostic and/or prognostic information.

A key limitation of several previous studies has been the lack of histologic confirmation of malignancy in patients treated as pancreas cancer. The present study provides a clear distinction between those patients in whom a histological diagnosis of cancer was confirmed and the cohort who were suspected of having pancreatic cancer but where the diagnosis was not confirmed. A further key feature of the study population in this study is that patients with obstructive jaundice were positively identified as not having pancreatobiliary malignancy at ERCP.

The combination of proven malignancy, proven non-malignant jaundice and follow-up until death make this a unique study and the largest to date that looks systematically through exploratory and challenge stages at the utility of Tu-M2-PK for diagnosis and prognosis in peri-ampullary cancer.

There were higher levels of both Tu-M2-PK and CA 19-9 in patients with pancreatic cancer compared to controls (table 1). Although Tu-M2-PK was less affected by jaundice than CA 19-9 (table 2), the relatively poor performance of both these tests indicates that they are of limited value even as adjunctive diagnostic tests in a tertiary care setting. Several studies have reported the utility of repeating an elevated CA 19-9 after relief of jaundice and our findings support this suggestion^{8,16,17}.

The exploratory survival analysis using a binary cut-off set at 27 U/ml for Tu-M2-PK provides important novel findings. Tu-M2-PK above 27 U/ml is a significant predictor of hazard of death (table 4) and this is observed in the Kaplan-Meier survival analysis (figure 2). This differential is maintained in the subgroup undergoing resection (figure 3).

The pathophysiologic basis of this finding is not known but as abnormalities of the aerobic glycolysis pathway are widespread in malignant cells, it appears that Tu-M2-PK functions as a metabolic marker of disseminated malignancy in this setting.

This finding can be readily integrated into current management pathways. Focusing on the tertiary care setting, the question of whether to undertake resection is increasingly based on the outcome of multidisciplinary discussion. This decision is particularly problematic in more elderly patients, those with co-morbidity and individuals with solid tumours of the head of the

pancreas of 3 – 4 cm in maximal transverse diameter where the probability of achieving curative or R₀ resection is low. In this latter group, the risk of microscopic dissemination of malignancy is high.

The findings of the present study require further verification by other workers, in particular the results would be substantiated by investigating the response of serial Tu-M2-PK levels in patients undergoing R₀ resection and the relation of Tu-M2-PK to molecular genetic prognostic markers (Sarr MG – personal communication). These points notwithstanding, our results suggest that Tu-M2-PK carried out during the pre-resection assessment phase is potentially a valuable predictor of poor differentiation and adverse outcome and thus may prove to be a valuable addition to the management of patients with peri-ampullary cancer.

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Table 1: Demographics and test values

	Suspected Cancer				Controls		
	Confirmed			Not confirmed			
	All	Non-jaundiced	Jaundiced		All	Non-jaundiced	Jaundiced
N	76	38	38	28	67	44	23
Age (SD)	64.9 (12.3)	62.8 (13.2)	66.9 (11.0)	62.8 (12.4)	53.4 (19.2)	48.9 (15.9)	62.0 (22.3)
Sex (% male)	55%	53%	55%	53%	28%	23%	39%
Tu M2-PK							
Mean (SD)	40.5 (26.4)	35.9 (23.1)	45.2 (28.9)	37.7 (29.3)	29.9 (20.9)	12.7 (7.3)	34.6 (24.7)
Median	36	30	37	26	25	11	27
IQR	21 – 54	19 – 49	23 – 62	18 – 45	16 – 40	7 – 18	18 – 43
Range	0 – 105	0 – 105	8 – 105	5 – 105	0-105	4 – 35	0 – 105
CA 19-9							
Mean (SD)	2477 (5328)	1206 (3083)	3749 (6687)	1513 (3770)	1017 (4011)	23.3 (48.3)	3003 (6602)
Median	163	34	1127	227	12	7	140
IQR	24 – 2244	10 – 341	129 – 2503	33 – 1438	5 – 41	4 – 20	12 – 1542
Range	0 – 25000	0 – 16177	0 – 25000	5 – 19520	0 – 23420	0 – 219	0 – 23420

Table 2: Sub-group analyses

<i>Effect of jaundice on diagnostic accuracy</i>						
	All		Non-jaundiced		Jaundiced	
	AOC	p	AOC	p	AOC	p
Tu-M2-PK	0.623	.012	0.607	.099	0.605	0.18
CA19-9	0.740	<.001	0.760	<.001	0.651	.053

<i>Sub-group analysis: prognostic disease markers</i>						
	Metastatic disease		Poor differentiation		Moderate-poor differentiation	
	AOC	p	AOC	p	AOC	p
Tu-M2-PK	0.680	.015	0.655	.035	0.574	.196
CA19-9	0.633	.071	0.429	.910	0.531	.591

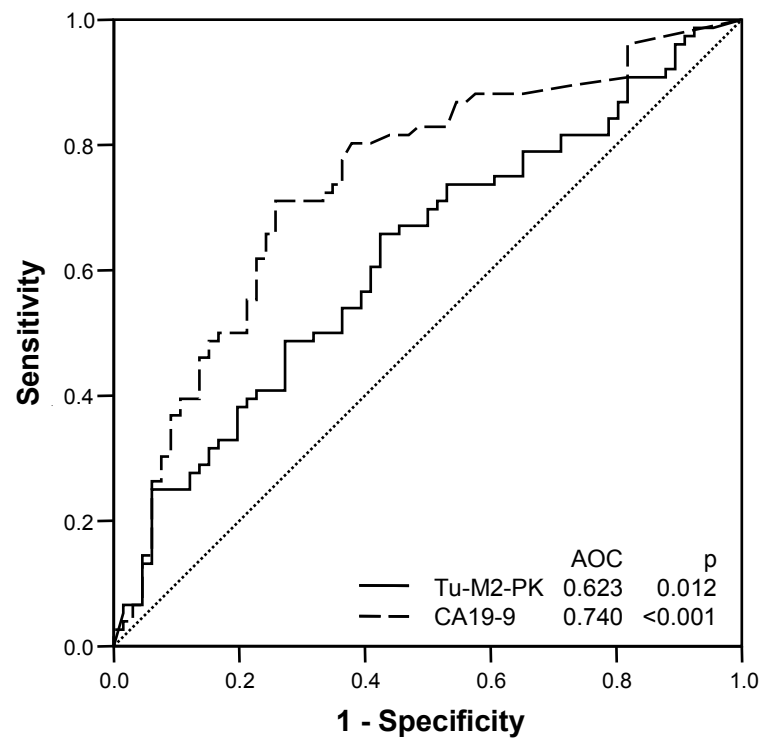
Table 3: Pearson correlation coefficients for survival analysis explanatory variables†

	age	sex	Metastatic disease	Poor differentiation	Tu-M2-PK	CA 19-9
age	1	.355***	.087	-.112	.208*	.210*
sex	.355***	1	.137	.137	.157	.137
Metastatic disease	.087	.137	1	.272***	.203*	.263**
Poor differentiation	-.112	.137	.272***	1	.208*	-.101
Tu-M2-PK	.208*	.157	.203*	.208*	1	.169
CA 19-9	.210*	.137	.263**	-.101	.169	1
† N=76 patients * significant at the 0.1 level (2-tailed) ** significant at the 0.05 level (2-tailed). *** significant at the 0.01 level (2-tailed).						

Table 4: Exploratory survival analysis

OUTCOME = Hazard of Death						
Dependent Variable = Survival from time of test.						
Initial Log Likelihood function: -2 Log likelihood (-2LL): 362.0						
	B	SE	Wald	Df	Sig.	Exp(B)
Base Model						
age	.017	.014	1.605	1	.205	1.018
sex	-.223	.320	.486	1	.486	.800
-2LL: 361.1; Chi-square: 1.7; DoF: 2 p: 0.43						
Incremental model: Metastatic disease						
age	.017	.014	1.486	1	.223	1.017
sex	-.226	.320	.498	1	.481	.798
MetaD	.193	.337	.326	1	.568	1.212
-2LL: 360.8; χ^2 : 0.3; DoF: 1 p: 0.58						
Incremental model: Poorly differentiated tumour						
age	.023	.014	2.564	1	.109	1.023
sex	-.382	.334	1.312	1	.252	.682
PoorD	.814	.328	6.158	1	.013	2.257
-2LL: 355.5; χ^2 : 7.4; DoF: 1 p: 0.018						
Incremental model: CA19-9						
age	.021	.014	2.155	1	.142	1.021
sex	-.249	.323	.594	1	.441	.779
CA19-9	.000	.000	.793	1	.373	1.000
-2LL: 360.2; χ^2 : 0.9; DoF: 1 p: 0.34						
Incremental model: Tu-M2-PK						
age	.005	.014	.110	1	.741	1.005
sex	-.171	.313	.298	1	.585	.843
Tu-M2-PK	.021	.006	11.030	1	.001	1.021
-2LL: 350.4; χ^2 : 10.7; DoF: 1 p: 0.001						
Incremental model: Tu-M2-PK (binary variable, cut off 27 U/ml)						
age	.017	.013	1.604	1	.205	1.017
sex	-.333	.319	1.091	1	.296	.717
Tu-M2-PK ⁺	.958	.361	7.039	1	.008	2.606
-2LL: 352.8; χ^2 : 8.3; DoF: 1 p: 0.004						

Figure 1: Receiver operating characteristic for cancer and non-cancer patients.



AOC: Area under curve

p: Non-parametric, asymptotic significance
(Null hypothesis: true area = 0.5)

Diagonal segments are produced by ties.

Figure 2: Kaplan Meier survival curve showing patients with high and low Tu-M2-PK test values.

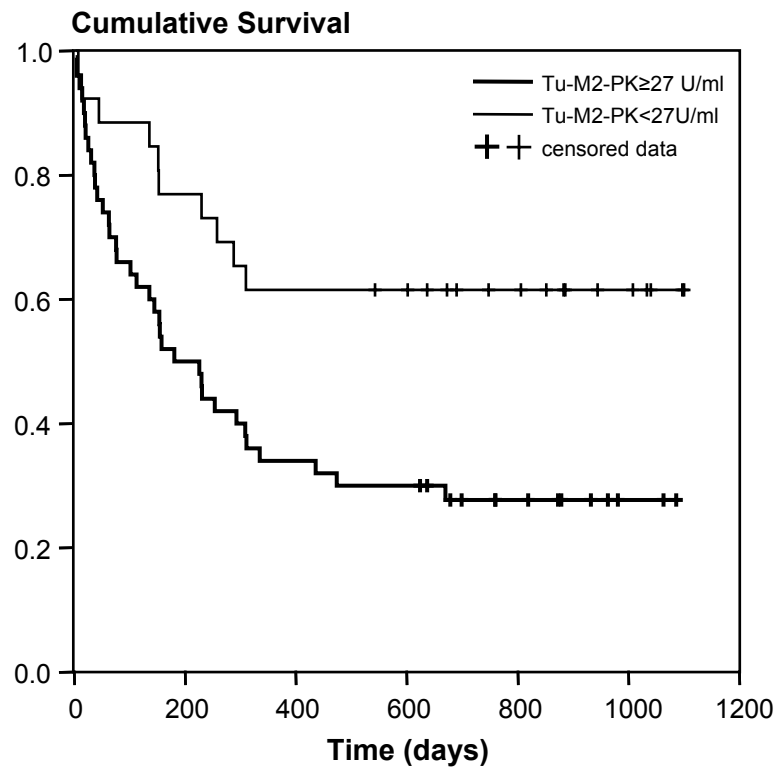


Figure and Table legends

Table 1

Demographic profile of patients in the study.

Table 2

Sub-group analyses. The diagnostic phase analysis examining the effect of jaundice is shown in patients with known cancer. The prognostic phase results examining patients with metastatic disease and poorly differentiated malignancy is shown.

Table 3

Pearson correlation coefficients for survival analysis.

Table 4:

Exploratory survival analysis.

The abbreviation poor D stands for poorly differentiated disease and the abbreviation meta D for metastatic disease. DoF = degrees of freedom.

Figure 1

Receiver operator characteristic for cancer and non-cancer patients.

Figure 2:

Kaplan-Meier survival curve for patients with Tu-M2-PK above 27 U/ml and those with Tu-M2-PK below 27 U/ml.

Figure 3:

Kaplan-Meier survival curve in patients undergoing resection with Tu-M2-PK above 27 U/ml and those with Tu-M2-PK below 27 U/ml.